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Technical report

Sterilisation of explants and cultures with sodium dichloroisocyanurate

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Abstract

The effectiveness of Sodium dichloroisocyanurate as a disinfectant for micropropagated plants was assessed. Analysis of the microbial flora of micropropagated plants showed a wide range of bacteria with predominantly *Pseudomonas, Xanthomonas* and Actinomycetes. Sodium dichloroisocyanurate was highly stable both as pre-prepared tablets and as solutions maintained at room temperature. Sterilisation of a range of plants which were heavily contaminated with bacteria was examined. Phytotoxicity was generally low and restricted to old leaves and cut surfaces. Solutions of Sodium dichloroisocyanurate were more effective at high concentrations (5000 ppm) than a commercially available bleach for disinfection of shoot cultures. Sodium dichloroisocyanurate was also used at low concentrations (300 ppm) for longer periods (24 h–48 h) to disinfect shoot explants from the field, and was at least as effective for sterilisation as a combination of Mercuric Chloride and Calcium hypochlorite.

Abbreviations: NaDCC = Sodium Dichloroisocyanurate

1. Introduction

It has been suggested that the major single problem faced by all commercial micropropagation companies is that of chronic contamination caused by microorganisms [1]. Mites, thrips, fungi, yeasts and bacteria have all been reported as contaminants of plant tissue cultures [2–4]. Latent bacteria are a particularly serious problem since considerable spread and multiplication of bacteria can occur unseen, and phytosanitory inspections of *in vitro* cultures may lead to the rejection of whole shipments of plants [5].

There is no one single antibiotic which will effectively control microorganisms, and the use of antibiotics to rid cultures of bacterial contamination is problematical [6]: Far better is to eliminate the microorganisms prior to culture. A number of sterilisation treatments are available for plants, but these all suffer from phytotoxicity due in large part to the highly oxidising nature of the compounds [7] and the high concentrations which must be used to produce effective levels of active chlorine.

Sodium dichloroisocyanurate has potentially a far more potent sterilant action than sodium hypochlorite since the compound dissociates to maintain constant levels of hypochlorous acid in solution as it is used. High levels of active chlorine are also maintained at plant physiological pH. Dichloroisocyanurate has been used for disinfection of plant materials [8,9], and this may prove to be a better sterilant than conventional compounds.

In this paper we present the results of studies on the microbial flora associated with micropropagated plants, the stability of Sodium dichloroisocyanurate (NaDCC) tablets and solutions, their phytotoxicity and their effectiveness in elimination of the microbial contaminants from a wide range of cultured plants.

2. Materials and methods

2.1 *Chemicals*

NaDCC tablets were obtained from Medentech Ltd, White Mill Ind. Est., Wexford, Co. Wexford, Republic of Ireland. Antibiotics were from Duchefa Bichemi, Haarlem, The Netherlands. API strips were obtained from Bio Merieux SA. All other chemicals were purchased from Sigma Chemicals, Poole, Dorset, UK.

2.2 *Shoot cultures*

Oak (*Quercus robur* L.) explants were maintained by monthly subculture on Woody Plant Medium [11]. All other shoot cultures were maintained by monthly subculture on modified Murashige and Skoog Medium (M&S) [10].

Spatyphyllium petite: M&S plus 92mg l^{-1} $MgSO_4 \cdot 7H_2O$, 340 mg l^{-1} NaH_2PO_4, 0.5 mg l^{-1} indole acetic acid (IAA) and 0.2 mg l^{-1} benzyl amino purine (BAP).

Globba wintii: M&S plus 170 mg l^{-1} NaH_2PO_4, 80 mg l^{-1} adenine, 1 mg l^{-1} IAA and 0.3 mg l^{-1} BAP.

Calathea exotica: M&S plus 170 mg l^{-1} NaH_2PO_4, 20 mg l^{-1} adenine, 100 mg l^{-1} casein enzymic hydrolysate, 1 mg l^{-1} naphthalene acetic acid and 3 mg l^{-1} BAP.

Anthurium var Southern blush: M&S plus 1 mg l^{-1} 2-isopentenyl adenine.

2.3 *Determination of microbial flora*

Microbial flora of shoot cultures was assayed by conventional microbiological techniques. API strips (API 20NE, API 50CHL and API Staph) were used to key out bacteria to the species level.

2.4 *Assessment of the stability of NaDCC*

The stability of NaDCC tablets and solutions was assessed by determination of available chlorine by titration. Samples of solutions were made up to a titratable volume with distilled water and 5 ml of glacial acetic acid added. 10 ml of 20% w/v potassium iodide was then added and the iodine liberated titrated with 0.1 M sodium thiosulphate to a pale yellow colour. 1 ml of 1% w/v starch was then added as an indicator and the titration continued to a clear end point.

NaDCC tablets were stored for up to one year under a variety of conditions. Samples were periodically dissolved in distilled water, immediately assessed for available chlorine and this expressed as a percentage of that of freshly manufactured tablets.

The effect of temperature on the stability of solutions of NaDCC was assessed by incubation of solutions containing two concentrations of available chlorine at 18 °C, 28 °C and 50 °C. The level of available chlorine was then determined at 0 h, 24 h and 48 h.

The long term stability of solutions of NaDCC was assessed. Solutions containing between 200 ppm and 10,000 ppm of available chlorine were incubated for up to 88 days at room temperature and the concentration of available chlorine measured periodically.

2.5 *Sterilisation Protocols*

The phytotoxicity of a range of disinfection treatments on shoot cultures of *Spatyphyllium* petite was assessed. Heavily contaminated explants were subcultured onto medium containing either 100ppm NaDCC or a cocktail of antibiotics filter sterilised into the culture media. The growth rate and quality of the main and side shoots were estimated at 28 days after subculture.

The efficiency of sterilisation was assessed by sterilisation for 15 min or 30 min of shoot cultures of *Spatyphyllium* petite by NaDCC or a commercially available solution of sodium hypochlorite (Domestos), both containing 5000 ppm available chlorine. The explants were then either transferred directly to culture media, or rinsed in 3 changes of sterile distilled water for 3 minutes prior to transfer. They were then cultured for 28 days and scored for the presence of microorganisms.

The efficiency of sterilisation of low concentrations of NaDCC was assessed on explants from field grown plants of oak. Solutions for sterilisation had 0.2% w/v Tween 20 added as a surfactant prior to sterilisation. Closed buds of oak shoots were excised with sterile forceps and scalpel and subjected to two types of sterilisation treatment. NaDCC was used at 5000 ppm for 30 min or at 300 ppm for 24 h or 48 h. Buds were also sterilised by a 30s rinse in 85% ethanol followed by 5 min 0.1% $HgCl_2$. They were then rinsed 3 times for 3 min in sterile distilled water prior to 20 min 7% $Ca(OCl)_2$. The rinsing was then repeated. Sterilised explants were then cultured for 28 days and the number of contaminated and dead explants, and number of new shoots recorded.

Table 1. Microbial flora of micropropagated plants

Plant Species	Microorganism
Spatyphyllium	*Xanthomomonas maltophila*
Globba wintii	*Pseudomonas spp*
	Alcaligenes xylosoxidans
	Actinomyctes
Calathea exotica	*Lactobacillus plantarum*
	Actinomycetes
Anthurium var	Actinomycetes
Southern blush	*Micrococcus kristinae*

Table 2. The effect of storage conditions on available chlorine levels in NaDCC tablets

Storage Conditions	Time (days)	Remaining Cl
Sealed Container	365	100%
Open Container 80% relative humidity 39 °C	42	100%
Open Container 12 hr Cycle 25 °C ⇒ 49 °C	17	100%
Open Container 60 °C	5	100%

Table 3. Available chlorine levels (ppm) in solutions kept at a range of temperatures

Treatment	temp	0 h	24 h	48 h
A	RT	560.7	546.7	532.5
	28 °C	–	390.5	–
	50 °C	–	298.2	127.8
B	RT	1043.7	1022.4	1001.1
	28 °C	–	766.8	–
	50 °C	–	738.4	284.0

3. Results and discussion

3.1 *Microbial flora of micropropagated plants*

Before sterilisation protocols can be effectively used, it was necessary to establish the microbial flora associated with the micropropagated plants. 86 isolates were recovered from 30 plants (Table 1). *Pseudomonas, Xanthomonas* and Actinomycetes predominated.

These bacteria may have arisen from endemic contaminants but some for example *Micrococcus kristinae* are predominantly associated with the skin of humans and other mammals and are known as introduced contaminants. They appear to be quite common contaminants of micropropagated plants [12].

None of these bacteria is regarded as a plant pathogen in the field, however at least one of these bacteria (*Lactobacillus plantarum*) is known to be strongly deleterious to the growth of micropropagated plants [13] and all decrease growth rates due to depletion of culture nutrients.

Antibiotics have been used successfully to eliminate microbes from cultured plants but this cannot be relied upon as bacteria may be resistant to commonly used antibiotics [14].

However, bacteria are sensitive to low concentrations of chlorine [15].

3.2 *Stability of Dichloroisocyanurate Tablets*

The levels of available chlorine in NaDCC tablets are stable for extensive periods (Table 2). This provides a great advantage over conventional bleaching solutions which must be purchased regularly or made up as fresh solutions from stocks on a regular basis.

3.3 *Stability of dichloroisocyanurate solutions*

Solutions of NaDCC lose their available chlorine rapidly at elevated temperatures (Table 3). The calculated half life of available chorine decreases from 30 days at room temperature to only one day at 50 °C. This suggests that solutions should be stored at low temperatures if not needed immediately.

Solutions of NaDCC at a range of concentrations are highly stable at room temperature (Figure 1). The calculated half life of available chlorine in these solutions is a minimum of 45 days.

There are many types of chlorine compounds which could be used for sterilisation studies, and it is important that the solutions are easy to use, and reproducible. The results show that NaDCC tablets can be stored for extended periods, and that solutions are very stable at room temperature with a half life of over a month. These results are consistent with those recorded previously [15] which showed high stability and low batch to batch variability of NaDCC products. This is in contrast to the inherent variability of solutions containing NaOCl.

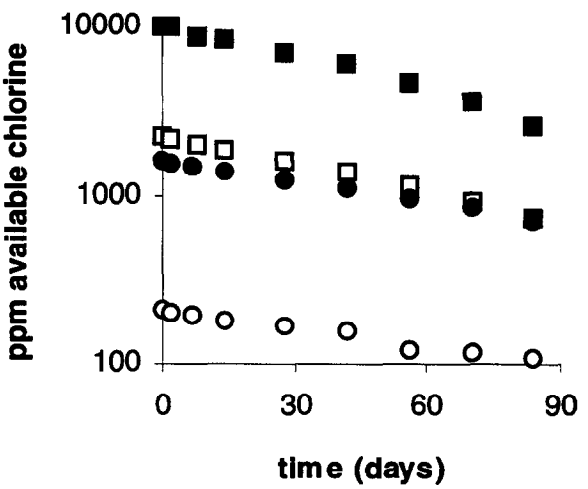

Figure 1. Available chlorine levels in solutions of NaDCC. NaDCC solutions were made up to 10,000 ppm (■), 2,000 ppm (□), 1,400 ppm (●) or 200 ppm (○), and stored at room temperature for up to 88 days. Available chlorine was measured periodically.

Table 4. Phytotoxicity of four disinfection regimes (A-D) on shoot cultures of *Spatyphyllium* petite

Tmt	Growth rate	Quality of main shoot	Quality of side shoot
1	3	5	4
2	2	1	2
3	2	1	2
4	5	5	5

Shoot cultures were subcultured onto media containing one of four disinfection regimes and scored on a semi-quantitative basis 28 days after subculture: 1 = poor → 5 = Excellent.
Key
All treatments were included in the medium.
Treatment A: 100 ppm Dichloroisocyanurate.
Treatment B: 5mg/l tetracycline; 20 mg/l polymixin; 40 mg/l vancomycin; 40 mg/l cefotaxime; 50 mg/l oxytetracycline.
Treatment C: Double treatment 2.
Treatment D: As treatment 3 but without oxytetracycline.

3.4 *Phytotoxicity*

Excellent disinfection was observed in many tissues which are sensitive to sodium and calcium hypochlorite (Table 4). Oxytetracycline was strongly phytotoxic, but plants performed almost as well in culture with NaDCC in the medium as with an antibiotic cocktail.

A rational strategy for elimination of bacterial contaminants from micropropagated plants relies on a detailed knowledge of their associated microbial flora and the antibiotic sensitivity [14]. Without such knowledge, elimination of bacteria by antibiotics is generally inefficient with a reduction in bacterial numbers due to bacteriostatic action while the antibiotic is present [6]. This has serious consequences for micropropagation and can result in the contamination of a shipment of plants once the antibiotic has been removed, or worse, at a later stage of propagation. Our results show that NaDCC does not have a high toxicity to plants, and may therefore be a useful sterilant. Both the growth rate and the quality of the main and side shoots in the NaDCC treated shoot cultures suggest that it has a useful disinfectant action.

Table 5. The effect of "Domestos" and NaDCC on the microbial flora associated with shoot cultures of *Spathiphyllum* petite

	Sterilant	Number of shoots	
		Total	Clean
No rinse	Domestos	38	0
No rinse	NaDCC	31	8
Rinse	Domestos	38	0
Rinse	NaDCC	32	2

Shoot cultures were sterilised for 15 min or 30 min with a solution of either "Domestos" or NaDCC containing 5000 ppm active chlorine. There was no significant difference in the proportion of clean cultures recovered with the two sterilisation times and the results have therefore been pooled. After sterilisation, cultures were either cultured immediately or rinsed in distilled water prior to culture. Clean shoots were those with no visible sign of contamination.

3.5 *Sterilisation efficiency*

The effects of NaDCC were compared to that of "Domestos" for sterilisation of heavily contaminated shoot cultures of *Spathiphyllum* petite (Table 5).

NaDCC gave significantly higher numbers of clean shoots than "Domestos". No clean explants were produced with "Domestos" whereas with NaDCC, 10 explants out of 63 were clear of contamination. Rinsing of explants after sterilisation significantly reduced the number of clean shoots produced by NaDCC treatment.

Given that all explants were heavily contaminated with bacteria prior to sterilisation, the production of clean explants which did not show visible signs of microbial infection after prolonged subculture is very encouraging. Phytotoxicity was relatively low, and tended to be associated with cut surfaces and old leaves. Most plants recovered after a period of slow growth. The low toxicity of NaDCC is an important factor in efficiency of sterilisation in that it permits culture

Table 6. Sterilisation of oak branch sprouts

Treatment	N	Contaminated	Dead	New shoots
0.1% $HgCl_2$ + 7% $Ca(OCl)_2$	37	3	10	23
0.5 hr * 5000 ppm NaDCC	13	0	4	5
24 hr * 300 ppm NaDCC	10	0	2	5
48 hr * 300 ppm NaDCC	8	0	2	8

Shoot tips from field grown oak were sterilised by each of the sterilisation regimes, and the number of dead and contaminated (cont'd) explants, and the number of new shoots recorded at 28 days after culture initiation.

of shoots without rinsing, and hence higher levels of sterilant are maintained for longer.

3.6 *Sterilisation at shoot explants at low concentrations of available chlorine*

Dichloroisocyanurate was examined at a lower range of concentrations for a longer period for the sterilisation of oak branch sprouts (Table 6). Some explants were killed by both sterilisation treatments and yellowing of the cut surfaces was apparent in all treatments. However, NaDCC did succeed in sterilisation of the explants. There were no significant differences in the proportion of dead or contaminated cultures, or the proportion of shoots produced between the totals for NaDCC and for Mercuric chloride plus Calcium hypochlorite.

Oak in common with many woody species is notoriously difficult to sterilise, and endemic contaminants are a common problem. Mercuric chloride in combination with calcium hypochlorite is the usual sterilant, and has been proven to be effective [16]. In our experiments, NaDCC was at least as effective as the conventional treatment. It must be borne in mind that the sterilisation protocol involving mercuric chloride and calcium hypochlorite has been optimised over a number of years to maximise sterilisation and minimise killing of shoots by the sterilisation treatment. Given that 100% sterilisation was observed with NaDCC it is probable that NaDCC concentrations were superoptimal and that a reduction in NaDCC concentration could increase recovery of shoots through a reduction in death of shoots. In our laboratory, we have successfully sterilised oak somatic embryo cultures with from 10–300 ppm NaDCC (results not shown) suggesting that the effective concentration for sterilisation could be much lower than 300ppm.

The toxicity of mercuric chloride is a particular problem [17] and presents intractable problems for disposal. These problems preclude its use on a large scale for use in micropropagation laboratories. Zero contamination rates were found with NaDCC and this may therefore prove to be a satisfactory alternative to sterilisation treatments involving mercury.

One possible problem with NaDCC is that of slow biodegradation. However, the product of NaDCC degradation, cyanuric acid, can be used as a source of nitrogen by bacteria in soil, sewage, river water and activated sludge. Degradation is particularly rapid under anaerobic or micro-aerophilic conditions where 95–100% of cyanuric acid may be degraded within hours [18]. The products of degradation are ammonia and CO_2 [19]. NaDCC is therefore an attractive candidate to replace the use of mercuric chloride without the problems of toxicity.

Rigorous proof of sterility in plant cell and tissue cultures is hard to obtain given the highly specific culture and environmental conditions required by some microorganisms. It has been said that the achievement of completely axenic cultures is unreasonable given the extreme hardiness of some bacteria, and that a more effective approach to plant tissue culture is to grow plants under non-axenic conditions [20].

We have shown that culture contamination levels can be reduced in a range of plant cultures to permit growth in culture where they would otherwise have had to be discarded; that it may be used for initial sterilisation of explants in preference to toxic sterilants; and that it may replace antibiotic addition to the medium as a method for controlling bacterial infection. NaDCC is used routinely in our laboratories for disinfection of shoot cultures and more than 50 species of plants have been successfully disinfected using this technique.

Future studies are necessary to examine the extent of microbial elimination by NaDCC as opposed to bacteriostatic action, and this will require rigorous examination of the microbial flora before and after sterilisation.

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